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state that the attached document is true and complete translation to the best of my knowledge of Japanese Patent Application No. 207508/1995.

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【File name】 SPECIFICATION

【Title of the Invention】

NOVEL PROTEINS AND METHODS FOR PRODUCING THE PROTEINS

【Claims】

【Claim 1】 A protein characterized by the following physicochemical properties and having biological activity to inhibit osteoclast differentiation and/or maturation.

(a) molecular weights on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions

(b) affinity; affinity to cation-exchanger and heparin.

(c) thermostability

; its biological activity to inhibit osteoclast differentiation and/or maturation is decreased by heating at 70℃ for 10 min. or at 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

(d) amino acid sequence

; internal amino acid sequences provided in sequence Nos. 1-3.

【Claim 2】 The protein according to claim 1 characterized in produced by human fibroblast cells.

【Claim 3】 A method of producing the protein according to claim 1 or 2 characterized in by cultivating human fibro-

blast cells, purifying the cultured solution by adsorption and elution in ion-exchange column, heparin-column, affinity-column, and reversed phase-column chromatography.

【Claim 4】 The method of producing the protein according to Claim 3 by cultivating human fibroblast cells on a carrier of alumina ceramic pieces.

【Claim 5】 A cDNA encoding amino acid sequence provided in sequence No. 4.

【Claim 6】 A DNA that hybridizes to cDNA encoding an amino acid sequence provided by sequence No. 4 in the sequence table.

【Claim 7】 A cDNA shown by a base sequence shown by sequence No. 5 in the sequence table.

【Claim 8】 A protein expressed by a cDNA encoding the amino acid sequence provided in sequence No. 4 in the sequence table.

【Claim 9】 A protein having inhibitory activity to differentiation and/or maturation of osteoclast expressed with a cDNA encoding the amino acid sequence of 80% or more homogeneity with that of shown by sequence No. 8 in the sequence table.

【Claim 10】 A method for production of the protein having following physicochemical properties by gene engineering technique using a cDNA encoding amino acid sequence shown by sequence No. 4 in the sequence table .

(a) molecular weights on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions

(b) affinity; affinity to cation-exchanger and heparin column.

(c) thermostability; a biological activity to inhibit osteoclast differentiation and/or maturation

; its activity is decreased by heating at 70℃ for 10 min. or at 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

(d) amino acid sequences;

; amino acid sequence Nos. 1-3 in the sequence table as internal amino acid sequences.

【Detailed Explanation of the Invention】

【0001】

【Field of the Invention】

This invention relates to a novel protein exhibiting inhibitory activity on differentiation and/or maturation of osteoclast, that is osteoclast inhibitory factor (OCIF), and methods for producing the protein.

[0002]

[Description of the Prior Arts]

Human bones are always remodeling by the repeated process of resorption and reconstitution. In the process, osteoblasts and osteoclasts are considered to be the cells mainly responsible for bone formation and bone resorption, respectively. A typical example of disease caused by the abnormal progress of bone metabolism includes osteoporosis. The disease is known to be provoked by the condition in which bone resorption by osteoclasts exceeds bone formation by osteoblasts, but the mechanism of osteoporosis has not yet been completely elucidated. Osteoporosis causes pain in the bone and makes the bone fragile, leading to fracture. Since osteoporosis increases the number of bedridden old people, it has become a social issue with the increasing number of old people. Therefore, development of efficacious drugs for the treatment of disease are eagerly expected to be developed. Bone mass reduction caused by the abnormal bone metabolism is thought to be prevented by inhibiting bone resorption, improving bone formation, or improving the balanced metabolism.

【0003】

Bone formation is expected to be promoted by stimulating growth, differentiation, or activation of osteoblasts, or inhibition of growth, differentiation or activation of osteoclast. Many physiologically active proteins including cytokines stimulating the growth of osteoblasts are focused attention and active investigation have been carried out and reported, e.g. fibroblast growth factors (FGFs) [Rodan, S.B. *et al.*, Endocrinology, *121*, 1917 (1987)], insulin-like growth factor-Ⅰ (IGF-Ⅰ) [Hook, J.M. *et al.*, Endocrinology, *122*, 254 (1988)], insulin-like growth factor-Ⅱ (IGF-Ⅱ) [McCarthy, T. *et al.*, Endocrinology, *124*, 301 (1989)], activin A [Centrella, M., *et al.* Mol. Cell. Biol., *11*, 250 (1991)], transforming growth factor-β [Noda M., The Bone, *2*, 29 (1988)], vasculotropin [Varonique, M., *et al.*, Biochem. Biophy. Res. Comm., *199*, 380 (1994)] and bone morphogenetic protein (BMP) BMP-2 [Yamaguchi, A., *et al.*, J. Cell Biol., *113*, 682 (1991), OP-1; [Sampath, T.K. *et al.*, J. Biol. Chem., *267*, 20532 (1992), Kuntsen, R., *et al.*, Biochem. Biophys. Res. Commun., *194*, 1352 (1993) have been reported.

【0004】

On the other hand, cytokines which inhibits differentiation and/or maturation of osteoclast such as transforming

growth factor-β [Chenu, C. *et al.*, Proc. Natl. Acad. Sci., U.S.A. *85*, 5683 (1983)] and interleukin-4 [Kasano, K. *et al.* Bone Miner., *2*, 179 (1993)] have been reported, and calcitonin [Bone-Miner., *17*, 347 (1992)] macrophage colony-stimulating factor [Hattersley, G., *et al.* J. Cell. Physiol., *137*, 199 (1988)], interleukin-4 [Watanabe, K., *et al.* Biochem. Biophys. Res. Commun., *172*, 1035 (1990)], and interferon-γ [Gowen, M. *et al.*, J. Bone Miner. Res., *1*, 469 (1986)] have been reported to inhibit the resorption of bone by osteoclast.

【0005】

These cytokines are expected to be efficacious drugs for improving bone mass reduction by stimulating bone formation and/or by inhibiting bone resorption. Some cytokines such as insulin like growth factor-I and parosteosis factors are now investigated in clinical trials. Calcitonin is already used as a drug to care osteoporosis and diminishes pain in osteoporosis.

【0006】

【Problems to be solved by the Invention】

Examples of drug products now clinically utilized for the treatment of bone diseases and for shortening the treatment period are active vitamin D_3, calcitonin and its derivatives, hormones such as estradiol, ipriflavon and calcium preparations. However, these drug products do not provide

satisfactory therapeutic effects, and novel drug substances have been expected to be developed. As mentioned above, bone metabolism is controlled in the balance between bone resorption and bone formation. Therefore, cytokines which inhibits osteoclast differentiation and/or maturation are expected to be developed as drugs for the treatment of bone diseases such as osteoporosis. Therefore, the object of the present invention is to provide a novel osteoclastogenesis inhibitory factor and an effective process for the production thereof.

【0007】

【Method to solve the Problems】

The inventors have intensively searched for osteoclastogenesis inhibitory factors and have found a novel osteoclastogenesis inhibitory factor (OCIF) which inhibits differentiation and/or maturation of osteoclasts in human embryonic fibroblast IMR-90 (ATCC CCL186) conditioned medium.

The inventors have established a method for accumulating the protein in a high concentration by culturing IMR-90 cells using alumina ceramic pieces as cell matrices.

The inventors have also established an efficient method for isolating and purifying the protein, OCIF, from the IMR-90 conditioned medium using sequential column chromatographies, including ion-exchange, heparin, affinity, and re-

versed phase.

【0008】

Then, cloning of cDNA encoding the protein can be obtained on the basis of amino acid sequence information for the obtained natural protein. Furthermore, a method was established for the production of the protein having inhibitory activity to differentiation and/or maturation of osteoclast by gene technology using the cDNA.

This invention relates to a protein derived from human fetal lung fibroblast cells and characterized by the following properties.

(a) molecular weight on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions.

(b) high affinity to cation-exchange column and heparin column.

(c) biological activity to inhibit osteoclast differentiation and/or maturation

; its activity is decreased by heating at 70℃ for 10 min. or 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

The structure of the OCIF protein is different from the previously reported proteins that inhibit osteoclast forma-

tion.

The invention includes a method for obtaining isolated and purified OCIF protein, comprising; (1) culturing human fibroblasts, (2) applying the cultured medium to a heparin column to obtain the adsorbed fraction, (3) treating the eluate with anion exchange column to give non-adsorbed fraction, (4) purifying the OCIF protein using cation-exchange column, (5) purifying the OCIF protein eluate using heparin, affinity and reversed-phase columns.

The column treatment of the present invention provides similar efficacious purification of OCIF protein not only in treatment of cultured mixtures with heparin Sepharose column but also in batch treatment of cultured mixture with heparin Sepharose. The affinity column, preferably Cibacron blue column is prepared by combining Cibacron blue F3GA on a carrier of hydrophilic synthetic polymer (cellulose) and generally called blue column.

Furthermore, the present invention relates to an effective process for the production of aforementioned protein using cell culture on an alumina ceramic carrier as a culture medium.

【0009】

The OCIF protein of the present invention can be isolated from human fibroblast conditioned medium with high yield.

Isolation procedure of OCIF is based on ordinary techniques for purifying proteins from biomaterials, in accordance with physical and chemical properties of the OCIF protein. For example, concentration procedure includes ordinary biochemical techniques such as ultrafiltration, lyophilization, and salting out. Purification procedure includes combinations of several chromatographic techniques for purifying proteins such as ion-exchange chromatography, affinity chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, and preparative gel electrophoresis. Human fibroblast for production of OCIF protein, preferably IMR-90 (ATCC CCL 186) is used as a raw material. A method for producing the IMR-90 conditioned medium is preferably a process comprising, adhering human fetal lung fibroblast IMR-90 cells to alumina ceramic pieces in roller-bottles, using DMEM medium supplemented with 5% new born calf serum for the cell culture, and cultivating the cells in roller-bottles for seven to 10 days without rotation. 0.1% CHAPS (3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate) is preferably added to the buffer as a detergent in the purification steps of OCIF protein.

[0010]

The protein OCIF of the present invention is obtained by carrying out the process as follows: the cultured solution

is charged to a heparin column (heparin Sepharose CL-6B, Pharmacia) and eluted with 10 mM Tris-HCl buffer containing 2M NaCl at pH 7.5 to give heparin adsorptive OCIF fraction. The fraction is charged to Q/anion exchange column (HiLoad-Q/FF. Pharmacia) and the non-adsorbed fraction are collected to give heparin adsorptive basic OCIF fraction. The obtained OCIF active fraction is successively treated with S/cation exchange column (HiLoad-S/Hp, Pharmacia), heparin column (heparin-5PW, Tosoh), Cibacron blue column (blue-5PW, Tosoh), and reversed phase column (BU-300C4, Perkin-Elmer Corp.) for isolation and purification. The prepared fraction can be defined by the aforementioned characteristic features.

【0011】

The present invention further relates to the method to clone cDNA encoding the protein based on the amino acid sequence of natural protein and to obtain OCIF protein that has the activity to inhibit differentiation and/or maturation of osteoclasts by gene technology. That is, the OCIF protein is purified according to the method described in the present invention and is treated with endoprotease (for example, lysylendopeptidase). The amino acid sequences of the digested peptides are determined and the mixture of all oligonucleotides that can encode each internal amino acid sequence are synthesized.

The OCIF cDNA fragment is obtained by PCR (preferably reversed transcriptase PCR, RT-PCR) using the oligonucleotides mixtures described as primers. The full length OCIF cDNA is cloned from a cDNA library using the OCIF cDNA fragment as probes. The resultant OCIF cDNA is inserted into an expression vector to give an OCIF expression plasmid. The recombinant OCIF can be produced by expressing the OCIF cDNA in various cells or bacteria.

[0012]

The OCIF activity was determined according to the method of Kumegawa, M. *et al.*, [Protein, Nucleic acid, Enzyme, *34*, 999 (1989)] and Takahashi, N. *et al.* [Endocrinology, *122*, 1373 (1988)]. That is, about 17-day-old mouse bone marrow cells are used as target cells and the inhibitory activity of osteoclast cells in the presence of active vitamin D_3 (calcitriol) is determined by the inhibition of induction of tartaric acid resistant acid phosphatase activity.

[0013]

The OCIF protein of the invention is useful as a pharmaceutical ingredients for treating or improving decreased bone mass such as osteoporosis, decreases of bone mass such as abnormal bone metabolism. The OCIF protein is also useful as an antigen to establish immunological diagnosis of the diseases. Pharmaceutical preparations containing the OCIF protein as an active ingredients are formulated and can

be orally or parenterally administered. The preparation contains the OCIF protein of the present invention as an efficacious ingredient and is safely administered to human and animals.

Examples of the pharmaceutical preparations include compositions for injection, intravenous drip infusion, suppositories, nasal preparations, sublingual preparations, and for percutaneous absorption. The preparation for injection is a mixture of the OCIF protein with pharmacologically efficacious amount and pharmaceutically-acceptable carriers. The carriers are vehicles and/or activators which are generally added to ingredients for injection, e.g. amino acids, saccharides, cellulose derivatives and other organic and inorganic compounds. When the OCIF protein is mixed with the vehicles and/or activators to prepare injections, pH adjuster, buffer, stabilizer, solubilizing agent, etc. can be added if necessary.

【0014】

【EXAMPLES】

The present invention will be further explained by providing examples, however, the scopes of the invention are not restricted by these examples.

Preparation of a conditioned medium of human fibroblast IMR-90

Human fetal lung fibroblast IMR-90 (ATCC-CCL 186) cells were cultured on alumina ceramic pieces (80 g) (alumina: 99.5%, Toshiba Ceramic K.K.) in DMEM medium (Gibco BRL Co.) supplemented with 5% FCS and 10 mM HEPES buffer (500 ml/roller bottle) at 37℃ in the presence of 5% CO_2 for 7-10 days using 60 roller bottles (490 cm^2, 110 x 171 mm, Coning Co.) without rotating the bottles. The conditioned medium was harvested, and a fresh medium was added to the roller bottles. About 30 L of IMR-90 conditioned medium per batch culture was obtained. The conditioned medium was designated as sample 1.

[0015]

Assay method for osteoclast development inhibitory activity

Osteoclast development inhibitory activity of the present invention was assayed by measuring tartrate-resistant acid phosphatase (TRAP) activity according to the methods of M. Kumegawa *et al.* [Protein, Nucleic Acid, Enzyme, *34*, 999 (1989)] and N. Takahashi *et al.* [Endocrinology, *122*, 1373 (1988)]. Briefly, bone marrow cells obtained form 17-day-old ddy mouse were suspended in α-MEM (manufactured by GIBCO BRL Co.) containing 10% FBS, 2 x 10^{-8} M of active vitamin D_3 and 100 μl each of test sample were inoculated to each well of 96-well plate at a rate of 3 x 10^5 cells. The plates were incubated for seven days at 37℃ in humidified 5% CO_2. Cultures were further continued by replacing

160 µl of old medium with 160 µl of sample diluted in α-MEN containing 10 % FBS and 1 x 10^{-8} active vitamine D_3 on day three and day five after starting culture. On day seven, after washing the plates with phosphate buffered saline, cells were fixed with ethanol/acetone (1:1) for one min. at room temperature, and then osteoclast development was tested for tartaric acid resistant acid phosphatase activity using a kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease of TRAP positive cells was taken as an indication of an OCIF activity.

[0016]

Purification of OCIF

i) Purification on a column of heparin Sepharose CL-6B

The IMR-90 conditioned medium (ca. 90 L) (sample 1) was filtered with 0.22 µm membrane filter (hydrophilic Mili-disk, 2,000 cm^2, Milipore Co.), and was divided into three parts. Each part (30 L) was applied to a heparin Sepharose CL-6B column (5 x 4.1 cm) equilibrated with 10 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5. After washing the column with 10 mM Tris-HCl buffer, pH 7.5 at a flow rate of 500 ml/hr., heparin Sepharose CL-6B adsorbent protein fraction (900 ml) was eluted with 10 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl. This fraction was designated as sample 2.

【0017】

ii) Purification on a column of HiLoad-Q/FF

The heparin Sepharose adsorbent fraction (sample 2) was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, supplemented with CHAPS to a final concentration of 0.1%, incubated at 4℃ overnight, and divided into two parts. Each part was then applied to an anion-exchange column (HiLoad-Q/FF, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5 to obtain a non-adsorbed fraction (1,000 ml). This fraction was designated as sample 3.

【0018】

iii) Purification on a column of HiLoad-S/HP

The HiLoad-Q non-adsorbent fraction (sample 3) was applied to a cation-exchange column (HiLoad-S/HP, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl buffer containing 0.1% CHAPS, pH 7.5. After washing the column with 50 mM Tris-HCl buffer containing 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient of 0-1 M NaCl over 100 min. at a flow rate of eight ml/min. and fractions of each 12 ml volume were collected. Each 10 fraction of Nos. 1-40 was composed to form one portion. Each 100 μl of the four portions was tested for OCIF activity. OCIF activity was observed in fractions from 11-30 as shown in Figure 1 (++: OCIF activity inhibiting osteoclast

development more than 80%, +: OCIF activity inhibiting osteoclast development between 30% and 80%, and -: no OCIF activity). The fractions from 21-30 having higher specific activity were collected and designated as sample 4.

【0019】

iv) Purification on an affinity column of heparin-5PW (heparin-5PW)

One hundred and twenty ml of HiLoad-S fractions of Nos. 21-30 (sample 4) was diluted with 240 ml of 50 mM Tris-HCl buffer containing 0.1% CHAPS, pH 7.5, and applied to an affinity column of heparin-5PW (0.8 x 7.5 cm, Tosoh Corp.) which was equilibrated with 50 mM Tris-HCl buffer containing 0.1% CHAPS, pH 7.5. The adsorbed protein was washed with 50mM Tris-HCl, 0.1% CHAPS,pH 7.5, and eluted with a linear gradient of up to 2 M NaCl over 60 min. at a flow rate of 0.5 ml/min. and fractions of 0.5 ml each volume were collected. In each fraction, 50 μl, was tested for OCIF activity. A fraction (10 ml) eluted with NaCl concentration from 0.7-1.3 M NaCl was found to have OCIF activity and was designated as sample 5.

【0020】

v) Purification on an affinity column of blue 5PW (blue-5PW)

Ten ml of heparin-5PW fraction eluted with 0.7-1.3 M NaCl (sample 5) was diluted with 90 ml of 50 mM Tris-HCl buffer containing 0.1% CHAPS, pH 7.5 and applied to an affinity

column of blue-5PW (0.5 x 5.0 cm, Tosoh Corp.) which was equilibrated with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5. After washing the column with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient of up to 2 M NaCl over 60 min. at a flow rate of 0.5 ml/min., and fractions of 0.5 ml each volume were collected. Using 25 μl of the each fraction, OCIF activity was evaluated. Fraction Nos. 49-70 eluted with about 1.0-1.6 M NaCl was found to exhibit OCIF activity. Figure 2 (++: OCIF activity inhibiting osteoclast development more than 80%, +: OCIF activity inhibiting osteoclast development between 30% and 80%, and -: no OCIF activity).

【0021】

vi) Purification on a reversed phase column

One ml of the blue 5PW fraction obtained by collecting fraction Nos. 49-50 was acidified with 10 μl of 25% TFA (trifluoroacetic acid) and applied to a reversed phase C4 column (BU-300, 2.1 x 220 mm, Perkin-Elmer) which was equilibrated with 0.1% of TFA and 25% of acetonitrile. The adsorbed protein was eluted with a linear gradient of up to 55% acetonitrile over 60 min. at a flow rate of 0.2 ml/min., and each protein peak was collected (Fig. 3). One hundred μl of the each peak fraction was tested for OCIF activity. The peaks 6-7 were found to have concentration dependent OCIF activity. The results are shown in Table 1.

【0022】

【Table 1】

OCIF activity eluted from reversed phase column

Sample	Dilution rate			
	1/40	1/120	1/360	1/1080
peak 6	++	++	+	-
peak 7	++	+	-	-

[++: OCIF activity inhibiting osteoclast development more than 80%.
+: OCIF activity inhibiting osteoclast development between 30% and 80%, and
-: no OCIF activity.]

【0023】

Molecular weight of OCIF protein

The two protein peaks (6 and 7) with OCIF activity were subjected to SDS-PAGE under reducing and non-reducing conditions. Each 20 μl peak fraction was concentrated under reduced pressure and dissolved in 1.5 μl of a mixture of 10 mM Tris-HCl buffer, pH 8, containing one mM EDTA, 2.5% SDS and 0.01% bromophenol blue, and incubated at 37℃ overnight under non-reducing conditions or reducing conditions (with 5% of 2-mercaptoethanol). Each 1.0 μl of sample was then analyzed by SDS-PAGE with a gradient gel of 10-15% acrylamide (Pharmacia Co.) and an electrophoresis device, Phast System (Pharmacia Co.). The following molecular

weight marker proteins were used to calculate molecular weight: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD). After electrophoresis, protein bands were visualized by silver stain with Phast Gel Silver Stain kit (Pharmacia Co.). The results are shown in Fig. 4.

[0024]

A protein band with about 60 kD was detected in the peak 6 under both reducing and non-reducing conditions. A protein band with about 60 kD was detected under reducing conditions and a protein band with about 120 kD was detected under non-reducing conditions in the peak 7. Therefore, the protein in the peak 7 was considered to be a homodimer of the protein in the peak 6.

[0025]

Thermostability test of OCIF

Twenty μl of sample from the blue-5PW fractions Nos. 51-52 was mixed with 30 μl of 10 mM phosphate buffered saline, pH 7.2, and incubated for 10 min. at 70℃ or 90℃, or for 30 min. at 56℃. These treated samples were tested for OCIF activity according to the aforementioned method. The results are shown in Table 2.

【0026】

【Table 2】

Heat stability of OCIF

Sample	Dilution rate		
	1/300	1/900	1/2700
untreated	++	+	-
70℃, 10 min.	+	-	-
56℃, 30 min.	+	-	-
90℃, 10 min.	-	-	-

[++: OCIF activity inhibiting osteoclast development more than 80%.
+: OCIF activity inhibiting osteoclast development between 30% and 80%, and
-: no OCIF activity.]

【0027】

(5) Determination of internal amino acid sequence of OCIF protein

The each of composed two fraction from Nos. 51-70 obtained from blue-5PW column were combined to one ml, and each one ml of sample was acidified with 10 μl of 25% TFA, and was applied to a reversed phase C4 column (BU-300, 2.1 x 220 mm, by Perkin-Elmer Corp.) which was equilibrated with 25% of acetonitrile containing 0.1% of TFA. The adsorbed protein was eluted with a linear gradient of up to 55% acetonitrile over 60 min. at a flow rate of 0.2 ml/min., and the protein

fractions corresponding to peaks 6 and 7 were collected, respectively. The protein in each peak was applied to a protein sequencer (PROCISE 494, Perkin-Elmer Corp.). However, the N-terminal sequence of the protein in each peak could not be analyzed. Therefore, N-terminal of the protein in each peak might blocked. So, internal amino acid sequences of these proteins were analyzed. The protein in peak 6 or 7 was concentrated and 50 μl of 0.5 M Tris-HCl buffer, pH 8.5, containing 100 μg of dithiothreitol, 10 mM EDTA, 7 M guanidine-HCl and 1% CHAPS was added to the each sample protein, and the mixture was allowed to stand for reduction for four hrs. at room temperature and incubated overnight with 0.2 μl of 4-vinylpyridine in the dark at a room temperature. The each sample which was acidified with 1 μl of 25% TFA was applied to reversed phase C4 column (BU-300, 2.1 x 30 mm, Perkin-Elmer Corp.) equilibrated with 20% acetonitrile containing 0.1% TFA. The pyridylethylated protein was eluted with a linear gradient of up to 50% acetonitrile over 30 min. at a flow rate of 0.3 ml/min., and each protein peak was collected. The pyridylethylated protein was concentrated by centrifugation and dissolved in 25 μl of 0.1 M Tris-HCl buffer, pH 9, containing eight M urea and 0.1% Tween 80. Seventy three μl of 0.1 M Tris-HCl buffer, pH 9, and 0.02 μg of API (lysyl endopeptidase, Wako Pure Chemical Ltd.) were added to the sample, and incubated

at 37℃ for 15 hrs. The sample which was mixed with 1 μl of 25% TFA was applied to a reversed phase C8 column (RP-300, 2.1 x 220 mm, Perkin-Elmer Corp.) which was equilibrated with 0.1% TFA. The peptide fragments were eluted from the column with a linear gradient up to 50% acetonitrile over 70 min. at a flow rate of 0.2 ml/min., and each peptide peak was collected (Fig. 5). The amino acid sequence of each peptide fragment (P1-P3) was analyzed with a protein sequencer. The sequences of the peptides were shown in the Sequence Nos. 1-3, respectively.

【0028】

(6) Determination of OCIF cDNA sequence

i) Isolation of poly(A)$^+$ RNA from IMR-90 cells

About 10 μg of poly(A)$^+$ RNA was isolated from 1 x 10^8 cells of IMR-90 by using Phast Track mRNA isolation kit (Invitrogen) according to the manufacturer's instruction.

【0029】

ii) Preparation of mixed primers

The following two mixed primers were synthesized based on the amino acid sequences of two peptides (Sequence table, sequence Nos. 2 and 3) obtained above. All the oligonucleotides in the mixed primers No. 2F can code for the amino acid sequence from the sixth residue, glutamine (Gln) to the twelfth residue, leucine (Leu), in peptide P2. All the complementary oligonucleotides in the mixed primers No. 3R

can code for the amino acid sequence from the sixth residue, histidine (His), to the twelfth residue, lysine (Lys), in peptide P3. The sequences of the mixed primers No. 2F and No. 3R are shown below.

【0030】

Table 3

No. 2F

5'-CAAGAACAAACTTTTCAATT-3'

G G G C C GC

A

G

【0031】

No. 3R

5'-TTTATACATTGTAAAAGAATG-3'

C G C G GCTG

A C

G T

【0032】

iii) Amplification of OCIF cDNA fragment by PCR (Polymerase chain reaction)

Single stranded cDNA was synthesized using Superscript II cDNA synthesis kit (Gibco BRL) and 1 μg of poly(A)$^+$ RNA obtained in (6)-i as a template according to the manufacturer's instruction. The OCIF cDNA fragment was obtained by PCR using the cDNA template and the primers shown in (6)-ii. The PCR was performed with the conditions as follows;

10X Ex Taq Buffer (Takara Shuzo)	5	μl
2.5 mM solution of dNTPs	4	μl
cDNA solution	1	μl
Ex Taq (Takara Shuzo)	0.25	μl
sterile distilled water	29.75	μl
40 μM solution of primer No. 2F	5	μl
40 μM solution of primer No. 3R	5	μl

The above components of the reaction were mixed in a micro-centrifugal tube. PCR step at 95℃ for three min. followed by 30 cycles at 95℃ for 30 sec., at 50℃ for 30 sec. and at 70℃ for 2 min. and then, final step was performed at 70℃ for 5 min. A part of the reaction mixture was subjected to agarose gel electrophoresis and about 400 bp homogenous DNA fragment was obtained.

【0033】

(7) Cloning of the OCIF cDNA fragment amplified by PCR and determination of its DNA sequence

The DNA fragment obtained by (6)-iii was inserted in the pBluescript II SK- vector (Stratagene) using DNA ligation kit (Takara Shuzo) to transfer *E. coli* DH5α (Gibco BRL). The transformants were cultured and the plasmid containing the OCIF cDNA (about 400 bp) was purified using the commonly

used method. The base sequence of the OCIF cDNA in the plasmid was determined using Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer Corp.). The base sequence had an expected amino acid sequence containing 132 residues. The amino acid sequences were used to design the mixed primers were found in the N-terminal and C-terminal amino acid sequences of the internal amino acid sequences Nos. 2 and 3, respectively. In addition, the internal amino acid sequences of OCIF (sequence No. 1) was found in the amino acid sequence of 132 amino acid residues. Above results confirmed that cloned about 400 bp cDNA is a fragment of OCIF cDNA.

[0034]

(8) Preparation of the DNA probe

A plasmid inserted with about 400 bp OCIF cDNA fragment shown above was used as a template and subjected to PCR for amplification according to the conditions described in (6)-iii. The OCIF cDNA was amplified. Agarose gel electrophoresis gave a OCIF cDNA fragment of about 400 bp. The OCIF cDNA was labeled with [α ^{32}P]dCTP using Megaprime DNA labeling system (Amersham) and used as a probe for the screening of the full length OCIF cDNA.

[0035]

(9) Preparation of cDNA library

cDNA was prepared using Great Lengths cDNA synthesis kit

(Clontech), oligo (dT) primer, and 2.5 μg of poly(A)$^+$ RNA obtained in the (6)-i as a template according to the manufacturer's instruction, EcoR I-Sal I-Not I adaptor was ligated to the cDNA. The purified cDNA was fractionated by the size of cDNA and precipitated with ethanol and dissolved in 10 μl of TE buffer. The cDNA with the adaptor was inserted in λZAP EXPRESS vector (Stratagene), which was previously cleaved with EcoR I, using T4DNA ligase. The recombinant phage DNA containing the cDNA was *in vitro* packaged using Gigapack gold II (Stratagene) and recombinant λZAP EXPRESS phage was prepared.

【0036】

(10) Screening of recombinant phage

Recombinant phage obtained in (9) was infected to *E.coli* XL1-Blue MRF' (Stratagene) at 37℃ for 15 min. The infected *E. coli* cells were added to NZY medium containing 0.7% agarose at 50℃ and plated on the NZY agar medium. The plates were incubated at 37℃ overnight. Hybond N (Amersham) were attached to the surface of plates containing plaques for 30 sec. The filter was denatured in an alkaline solution, neutralized, and soaked in 2xSSC according to the standard protocol. The DNA was immobilized on the filter using UV crosslinker (Stratagene). The filter was incubated in the hybridization buffer (Amersham) containing 100 μ/ml salmon sperm DNA at 65℃ for four hrs. and then hybridized

overnight in the same buffer containing 2 x 10^5 cpm/ml thermally denatured DNA probe. The filter was washed twice with 2xSSC, and twice with a solution containing 0.1xSSC and 0.1% SDS at 65℃for 10 min. each time. The resultant positive clones were purified twice by screening. The purified clone containing about 1.6 kb insert was used in the experiments described below. This phage was infected to *E. coli* XL1-Blue MRF' according to the manufacturer's instruction of λZAP Express cloning kit (Stratagene). Then, multiply infected with a helper phage ExAssist (Stratagene). The broth containing the cultured supernatant was infected to *E. coli* XLOLR (Stratagene). The infected XLOLR was plated and kanamycin resistant strain was picked up. The transformant having the plasmid insert about 1.6 kb called pBKCMV was obtained. The transformant was deposited to National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Technology as FERM P-14998. The transformant was grown and plasmid was purified according to the standard protocol.

【0037】

(11) Determination of the base sequence of cDNA encoding for full amino acid sequence of OCIF

The base sequence of OCIF cDNA obtained in (10) was determined using Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer Corp.). The used primers were T3, T7 primers

(Stratagene) and synthetic primers designed according to the OCIF cDNA base sequence. The sequences of these primers are shown below.

T3; 5'-AATTAACCCTCACTAAAGGG-3'
T7; 5'-GTAATACGACTCACTATAGGGC-3'
IF1; 5'-ACATCAAAACAAAGACCAAG-3'
IF2; 5'-TCTTGGTCTTTGTTTTGATG-3'
IF3; 5'-TTATTCGCCACAAACTGAGC-3'
IF4; 5'-TTGTGAAGCTGTGAAGGAAC-3'
IF5; 5'-GCTCAGTTTGTGGCGAATAA-3'
IF6; 5'-GTGGGAGCAGAAGACATTGA-3'
IF7; 5'-AATGAACAACTTGCTGTGCT-3'
IF8; 5'-TGACAAATGTCCTCCTGGTA-3'
IF9; 5'-AGGTAGGTACCAGGAGGACA-3'
IF10; 5'-GAGCTGCCCTCCTGGATTTG-3'
IF11; 5'-CAAACTGTATTTCGCTCTGG-3'
IF12; 5'-GTGTGAGGAGGCATTCTTCA-3'

The determined base sequence of the OCIF is shown in the sequence No. 5 and the presumed amino acid sequence is shown in the sequence No. 4.

[0038]

(12) Production of expression plasmid of OCIF cDNA

The plasmid inserted with about 1.6kb OCIF cDNA obtained in (10) was digested with restriction enzymes, BamH I and Xho I. The OCIF cDNA insert was cut out, separated by an agarose gel electrophoresis, and purified using QIAEX DNA isolation kit. The purified OCIF cDNA insert was ligated using DNA ligation kit (Takara Shuzo) to the expression plasmid pCEP4 (Invitrogen) digested with restriction en-

zymes, BamH I and Xho I. *E. coli* DH5α (Gibco BRL) was transformed with the ligation mixture. The transformants were grown and the expression plasmid inserted with the OCIF cDNA was purified using QIAGEN column (QIAGEN). The OCIF expression plasmid was precipitated with ethanol. The precipitates were dissolved in sterile distilled water and used for the subsequent procedure.

[0039]

(13) Transient expression of OCIF cDNA and analysis of its biological activity.

Recombinant OCIF was produced using the expression plasmid, prepared in (12) according to the method described below and the activity was determined. 8×10^5 cells of 293/EBNA (Invitrogen) were inoculated in each well of the 6-well plate using IMDM containing 10% fetal calf serum (Gibco BRL). The culture medium was removed next day and the cells were washed with serum free IMDM. The OCIF expression plasmid diluted with OPTI-MEM (Gibco BRL), and lipofectamine (Gibco BRL) for transfection were mixed. The mixture was added to the cells in each well. Three μg of the expression plasmid and 12 μl of lipofectamine were used for each transfection. After the incubation for 38 hrs., the medium was replaced with one ml of fresh OPTI-MEM. After the transfected cells were incubated for 30 hrs., the conditioned medium was harvested and used for the assay of OCIF

activity. The OCIF activity of recombinant OCIF was analyzed according to the method described below. In 96-well microtiter plate, 100 μl of the sample diluted with α-MEM (Gibco BRL Co.) containing 10% FBS and 2×10^{-8} of active vitamin D_3 and 100 μl of a suspension of 3×10^5 bone marrow cells obtained from 17-days-old mice in α-MEM containing 10% FBS was inoculated and cultured for seven days at 37℃ in humidified 5% CO_2. During incubation, 160 µl of old medium in each well was replaced with 160 µl of the sample diluted in α-MEM containing 1×10^{-8} M active vitamin D_3 and 10% FBS on days three and five after the inoculation of cells. On day seven, after washing with phosphate buffered saline, cultures were fixed with ethanol/acetone (1:1) for one min. at room temperature, and then osteoclast development was tested for acid phosphatase activity using a kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease of TRAP positive cells determination kit was used for the staining and determination. OCIF activity was determined by the decrease of acid phosphatase positive cells. Similar OCIF activity to that of natural OCIF protein from IMR-90 conditioned medium was confirmed. (Table 3).

【0040】

Table 3. OCIF activity in 293/EBNA conditioned medium.

Cultured cell	Dilution						
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
OCIF gene transfected	++	++	++	++	++	+	-
vector transfected	-	-	-	-	-	-	-
untreated	-	-	-	-	-	-	-

[++: OCIF activity inhibiting osteoclast development more than 80%.
+: OCIF activity inhibiting osteoclast development between 30% and 80%, and
-: OCIF activity.]

【0041】

【Effect of the Present Invention】

A novel protein having inhibitory activity to osteoclast formation and a method of effective production of the protein are provided by the present invention. The protein of the present invention has inhibitory activity on osteoclast formation and is useful for the treatment of various dis-

eases such as bone mass reduction including osteoporosis and immunological diagnosis thereof.

[0042]

[Sequence table]

Sequence No: 1

Length of sequence: 6

Type of sequence: amino acids

No. of chain: 1

Topology: linear

Molecular type: peptide (internal amino acid of protein)

Sequence: Xaa-Tyr-His-Phe-Pro-Lys
1 5

[0043]

Sequence No: 2

Length of sequence: 14

Type of sequence: amino acids

No. of chain: 1

Topology: linear

Molecular type: peptide (internal amino acid of protein)

Sequence: Xaa-Gln-His-Ser-Xaa-Gln-Glu-Gln-Thr-Phe-Gln-Leu-
1 5 10
Xaa-Lys

[0044]

Sequence No: 3

Length of sequence: 12

Type of sequence: amino acids

No. of chain: 1

Topology: linear

Molecular type: peptide (internal amino acid of protein)

Sequence: Xaa-Ile-Arg-Phe-Leu-His-Ser-Phe-Thr-Met-Tyr-Lys
1 5 10

[0045]

Sequence No: 4

Length of sequence: 401

Type of sequence: amino acids

No. of chain: 1

Topology: linear

Molecular type: protein

Sequence:

Met Asn Asn Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser
1 5 10 15

Ile Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His
20 25 30

Tyr Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro
35 40 45

Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr
50 55 60

Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His
65 70 75

Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu
80 85 90

Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val Cys
95 100 105

Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys
110 115 120

His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr
125 130 135

Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe
140 145 150

Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
155 160 165

Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr
170 175 180

His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys
185 190 195

Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala
200 205 210

Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp
215 220 225

Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
230 235 240

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys
245 250 255

Leu Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile
260 265 270

Ile Gln Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile
275 280 285

Gly His Ala Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu
290 295 300

Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr
305 310 315

Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser
320 325 330

Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu
335 340 345

Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys Thr
350 355 360

Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe
365 370 375

Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly
380 385 390

Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
395 400

[0046]

Sequence No: 5

Length of sequence: 1206

Type of sequence: nucleic acids

No. of chain: 1

Topology: linear

Molecular type: cDNA

Sequence:

ATGAACAACT TGCTGTGCTG CGCGCTCGTG TTTCTGGACA TCTCCATTAA GTGGACCACC 60
CAGGAAACGT TTCCTCCAAA GTACCTTCAT TATGACGAAG AAACCTCTCA TCAGCTGTTG 120
TGTGACAAAT GTCCTCCTGG TACCTACCTA AAACAACACT GTACAGCAAA GTGGAAGACC 180
GTGTGCGCCC CTTGCCCTGA CCACTACTAC ACAGACAGCT GGCACACCAG TGACGAGTGT 240
CTATACTGCA GCCCCGTGTG CAAGGAGCTG CAGTACGTCA AGCAGGAGTG CAATCGCACC 300
CACAACCGCG TGTGCGAATG CAAGGAAGGG CGCTACCTTG AGATAGAGTT CTGCTTGAAA 360
CATAGGAGCT GCCCTCCTGG ATTTGGAGTG GTGCAAGCTG GAACCCCAGA GCGAAATACA 420
GTTTGCAAAA GATGTCCAGA TGGGTTCTTC TCAAATGAGA CGTCATCTAA AGCACCCTGT 480
AGAAAACACA CAAATTGCAG TGTCTTTGGT CTCCTGCTAA CTCAGAAAGG AAATGCAACA 540
CACGACAACA TATGTTCCGG AAACAGTGAA TCAACTCAAA AATGTGGAAT AGATGTTACC 600
CTGTGTGAGG AGGCATTCTT CAGGTTTGCT GTTCCTACAA AGTTTACGCC TAACTGGCTT 660
AGTGTCTTGG TAGACAATTT GCCTGGCACC AAAGTAAACG CAGAGAGTGT AGAGAGGATA 720
AAACGGCAAC ACAGCTCACA AGAACAGACT TTCCAGCTGC TGAAGTTATG GAAACATCAA 780
AACAAAGACC AAGATATAGT CAAGAAGATC ATCCAAGATA TTGACCTCTG TGAAAACAGC 840

GTGCAGCGGC ACATTGGACA TGCTAACCTC ACCTTCGAGC AGCTTCGTAG CTTGATGGAA 900

AGCTTACCGG GAAAGAAAGT GGGAGCAGAA GACATTGAAA AAACAATAAA GGCATGCAAA 960

CCCAGTGACC AGATCCTGAA GCTGCTCAGT TTGTGGCGAA TAAAAAATGG CGACCAAGAC 1020

ACCTTGAAGG GCCTAATGCA CGCACTAAAG CACTCAAAGA CGTACCACTT TCCCAAAACT 1080

GTCACTCAGA GTCTAAAGAA GACCATCAGG TTCCTTCACA GCTTCACAAT GTACAAATTG 1140

TATCAGAAGT TATTTTTAGA AATGATAGGT AACCAGGTCC AATCAGTAAA AATAAGCTGC 1200

TTATAA 1206

【Brief description of the drawings】

【Fig. 1】

Figure 1 shows the elution pattern of crude OCIF product (Hiload-Q/FF through fraction ; sample 3) from Hiload-S/HP column.

【Fig. 2】

Figure 2 shows the elution pattern of crude product (heparin-5PW fraction ; sample 5) from blue-5PW column.

【Fig. 3】

Figure 3 shows the elution pattern of blue-5PW eluted fractions 49 to 50 from reversed-phase column.

【Fig. 4】

Figure 4 shows the SDS-PAGE pattern of final product under reducing conditions or non-reducing conditions.

【Description of the lanes】

lanes 1,4 ; molecular weight marker proteins

lanes 2,5 ; OCIF protein of peak 6

lanes 3,6 ; OCIF protein of peak 7

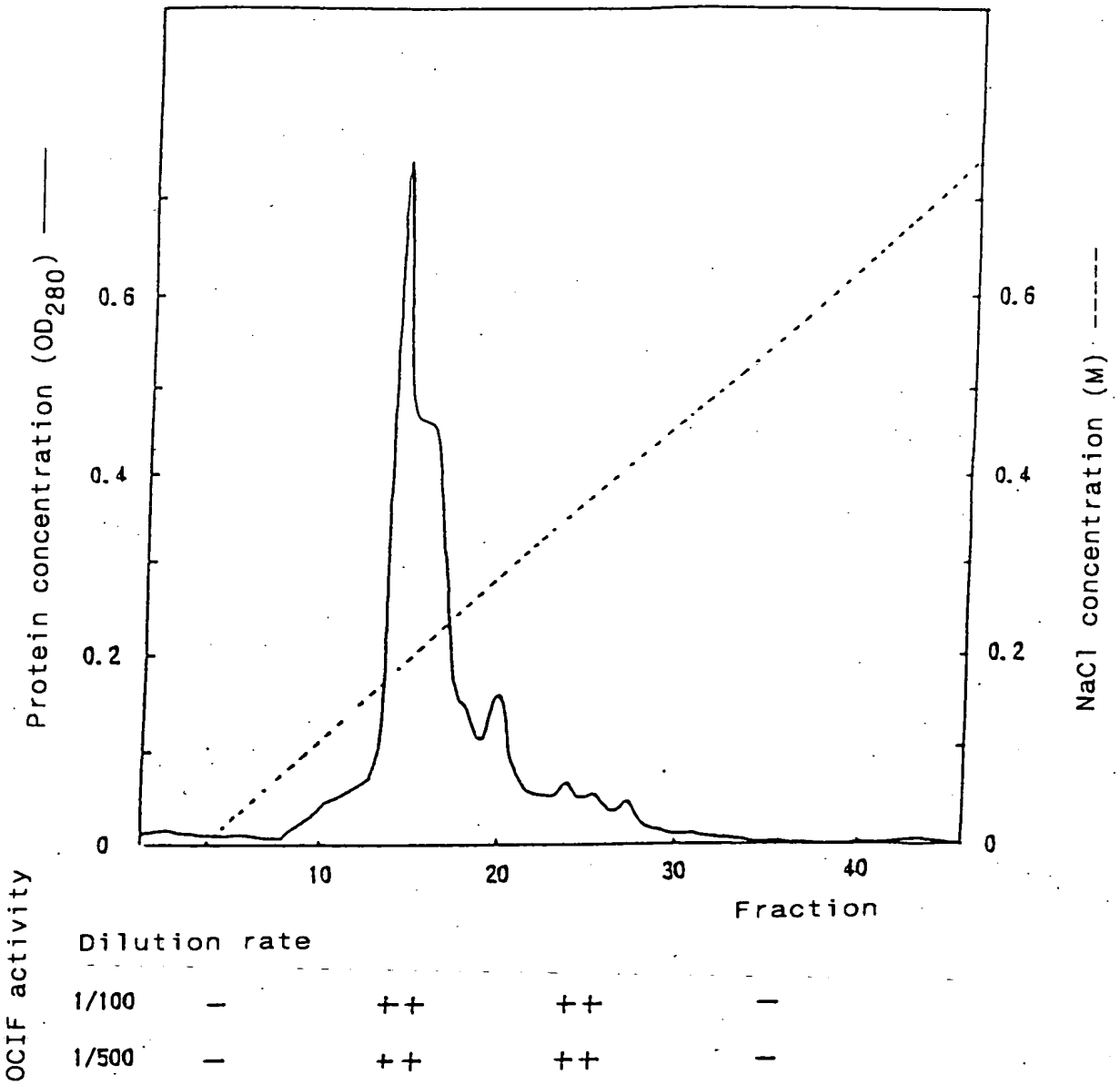
【Fig. 5】

Figure 5 shows the elution pattern of peak 7 after pyridylethylation under reducing condition followed by digestion with lysylendopeptidase on a reversed-phase column.

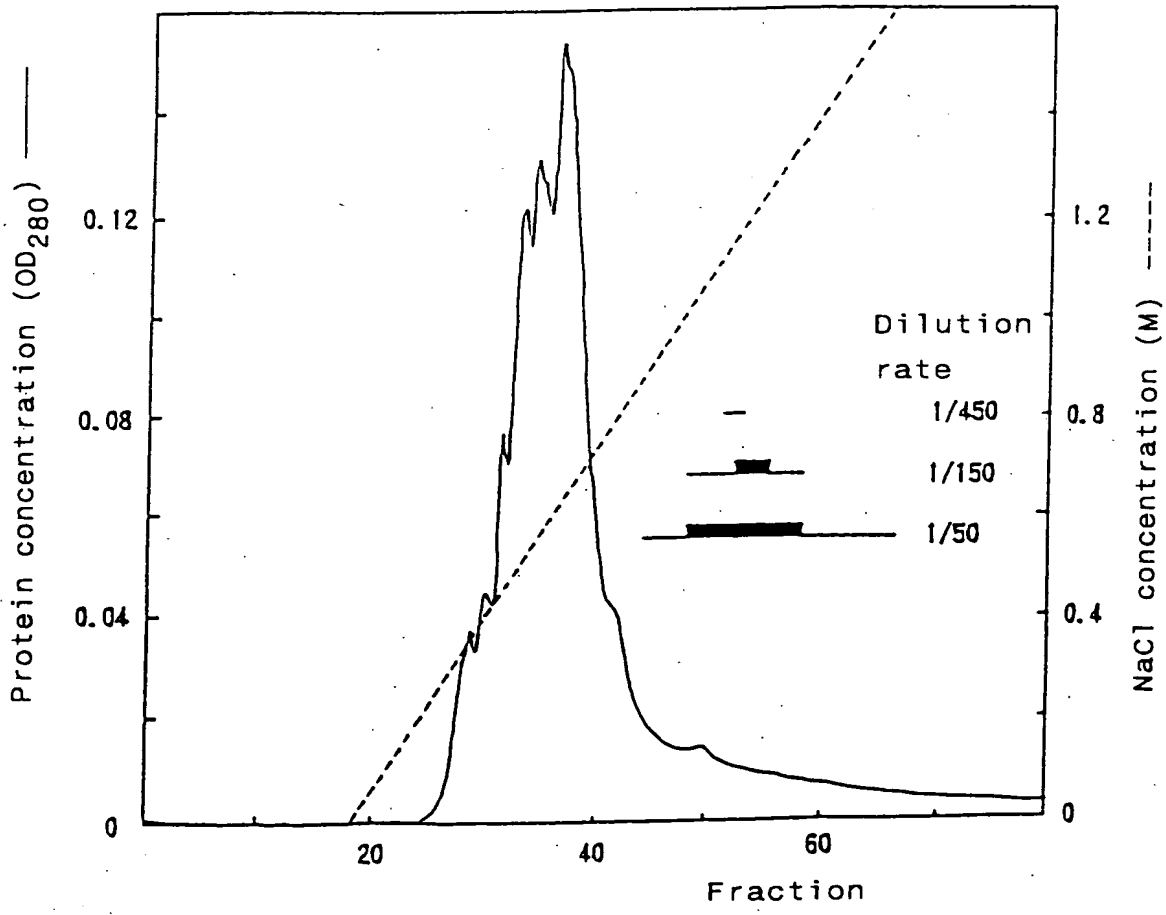
File No. SNMFP95239

[File name] Drawing

[Figure 1]

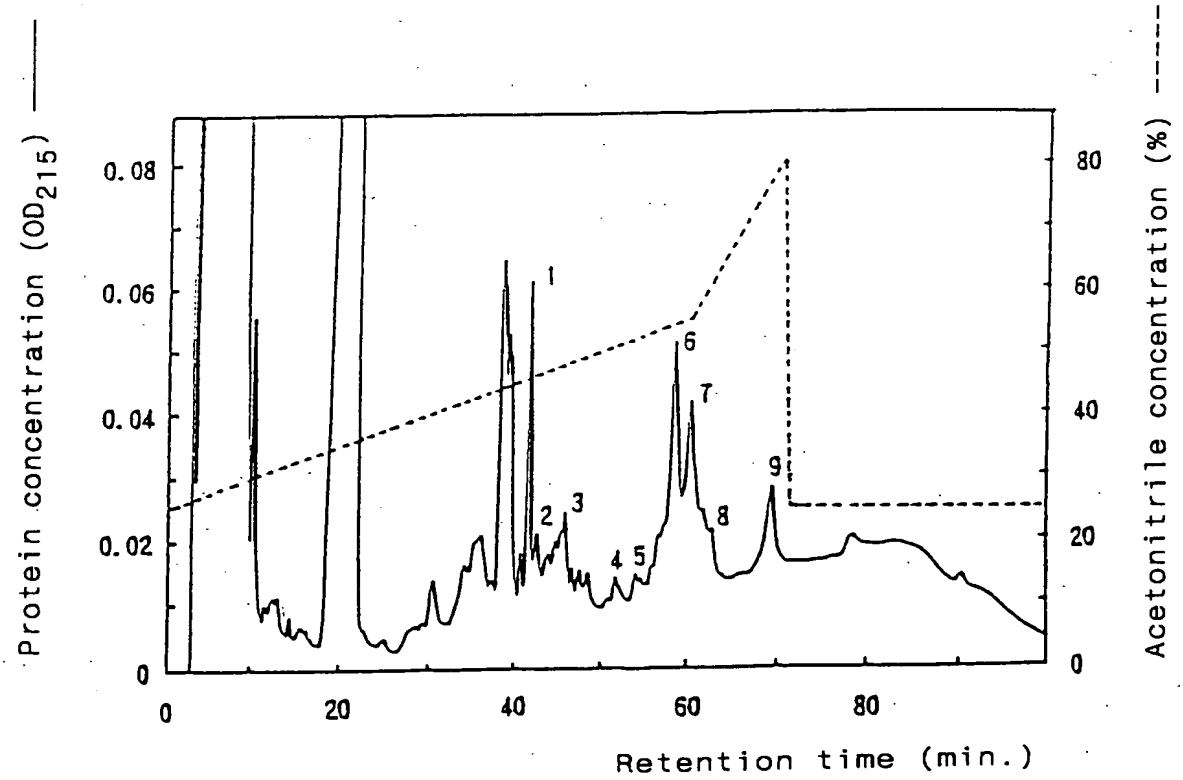


[Figure 2]

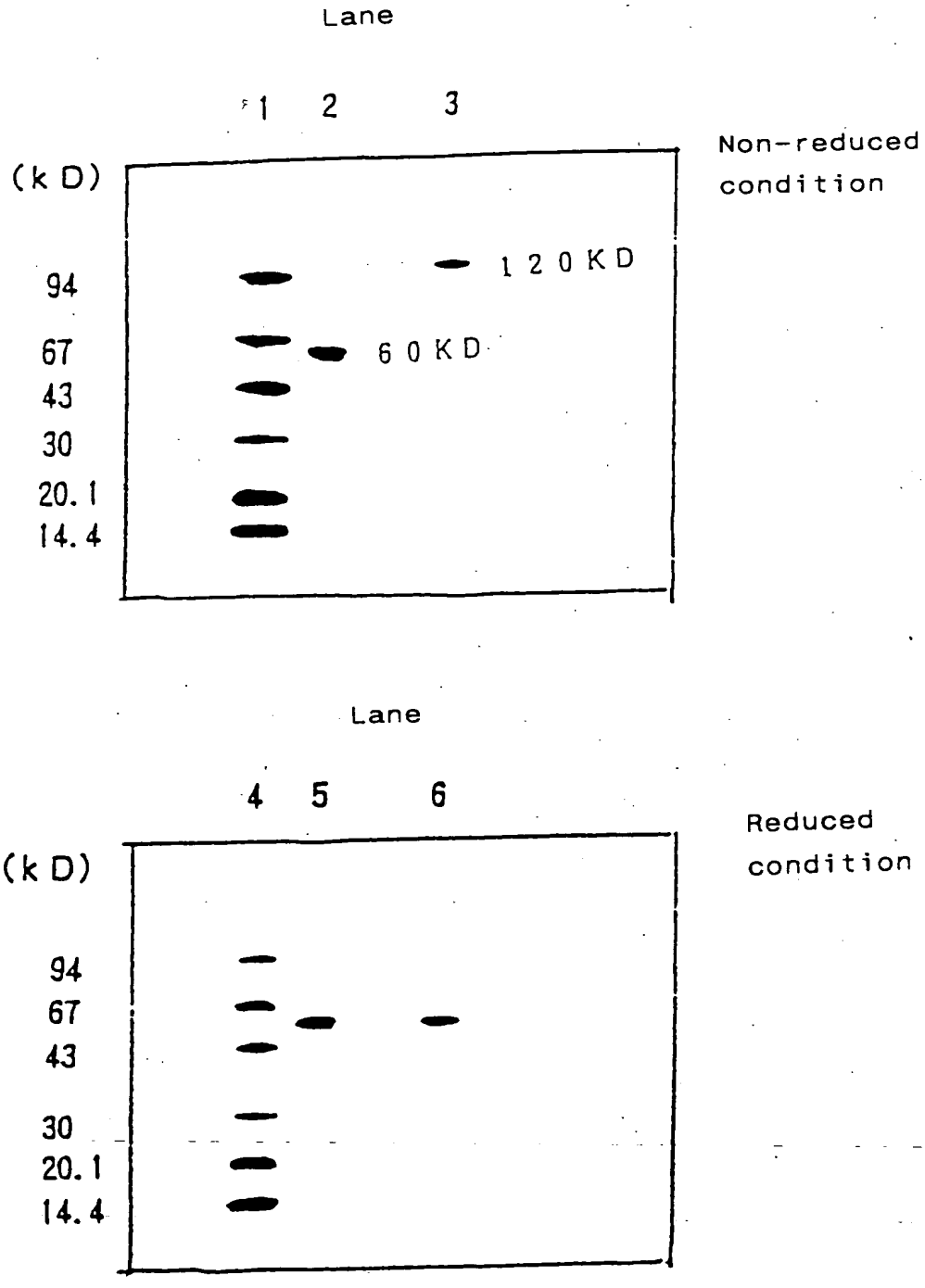


OCIF activity +: —— , ++: ▬

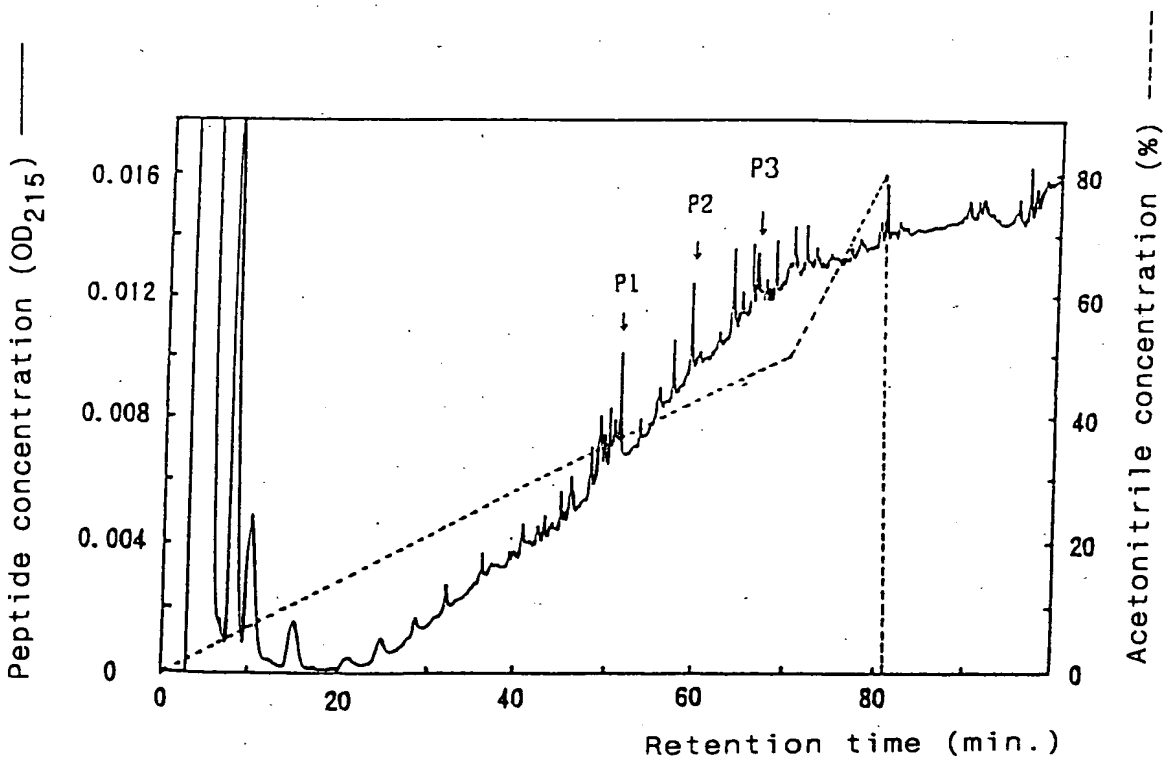
[Figure 3]


Protein concentration (OD_{215}) ——
Acetonitrile concentration (%) - - - -
0.08
0.06
0.04
0.02
0
80
60
40
20
0
0
20
40
60
80
Retention time (min.)
1
2
3
4
5
6
7
8
9

[Figure 4]


Lane
1 2 3
(k D)
94
67
43
30
20.1
14.4
120KD
60KD
Non-reduced condition
Lane
4 5 6
(k D)
94
67
43
30
20.1
14.4
Reduced condition

【Figure 5】


Peptide concentration (OD_{215}) ——
Acetonitrile concentration (%) - - - -
0.016
0.012
0.008
0.004
0
80
60
40
20
0
0
20
40
60
80
P1
P2
P3
Retention time (min.)

【File name】 Abstract

【Abstract】

【Construction】

A novel protein having inhibitory activity on osteoclast differentiation and/or maturation.

(a) molecular weights on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions

(b) affinity; affinity to cation-exchanger and heparin

(c) thermostability

; its biological activity to inhibit osteoclast differentiation and/or maturation activity is decreased by heating at 70℃ for 10 min. or at 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

(d) amino acid sequence

; internal amino acid sequences provided in sequence Nos. 1-3.

A method of producing the protein having inhibitory activity to differentiation and/or maturation of osteoclast prepared by application of cultured solution of human fibroblast cells for purification of the cultured solution by repeated absorption and elution in ion-exchange column,

heparin-column, affinity-column, and reversed phase-column chromatography.

【Effect】 The protein is useful for the treatment of bone mass reducing diseases such as osteoporosis or a biochemical test reagent.

【Selected figures】 No

File No. SNMFP95239
File form 7

CERTIFICATE OF ACCEPTANCE

Notice No.: Deposit No. 978 (1995)
Date of Notice: June 21, 1995

Mr. Yasuyoshi Takeshita
Manager, Bioscience Research Institute,
Snow Brand Milk Products Co., Ltd.

Michio Ohishi
Manager,
Agency of Industrial Science and Technology,
National Institute of Bioscience and Human-Technology

Ⅰ. Microorganism
Title for identification given by the depositor
pBK/01F10
Deposit No. FERM P-14998

Ⅱ. Scientific nature and position in taxonomy
Microorganism shown in column Ⅰ is attached the following document.

Scientific nature: No
Position in taxonomy: Yes

Ⅲ. Receipt and deposit

Agency of Industrial Science and Technology, National Institute of Bioscience and Human-Technology deposits the microorganism illustrated in column Ⅰ on June, 21, 1995.

207508-1995

[Title of the Document] DATA CORRECTION EX OFFICIO
[Document to be Corrected] Patent Application

<Recognized Information and Additional Information>
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207508-1995

HISTORICAL INFORMATION ON APPLICANT

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